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Calmodulin and ATP support activity of the Cav1.2 channel through dynamic interactions with the channel

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Key points

- Cav1.2 channels maintain activity through interactions with calmodulin (CaM). In this study, activities of the Cav1.2 channel (α1C) and of mutant-derivatives, C-terminal deleted ($\alpha 1C\Delta$) and $\alpha 1C\Delta$ linked with CaM ($\alpha 1C\Delta$CaM), were compared in the inside-out mode.
- $\alpha 1C\Delta$ with CaM, but not without CaM, and $\alpha 1C\Delta$CaM were active, suggesting that CaM induced channel activity through a dynamic interaction with the channel, even without the distal C-tail.
- ATP induced α1C activity with CaM and enhanced activity of the mutant channels. Okadaic acid mimicked the effect of ATP on the wildtype but not mutant channels.
- These results supported the hypothesis that CaM and ATP maintain activity of Cav1.2 channels through their dynamic interactions. ATP effects involve mechanisms both related and unrelated to channel phosphorylation.
- CaM-linked channels are useful tools for investigating Cav1.2 channels in the inside-out mode; the fast run-down is prevented by only ATP and the slow run-down is nearly absent.

Abstract Calmodulin (CaM) plays a critical role in regulation of Cav1.2 Ca^{2+} channels. CaM binds to the channel directly, maintaining channel activity and regulating it in a Ca^{2+}-dependent manner. To explore the molecular mechanisms involved, we compared the activity of the wildtype channel (α1C) and mutant derivatives, C-terminal deleted ($\alpha 1C\Delta$) and $\alpha 1C\Delta$ linked to CaM ($\alpha 1C\Delta$CaM). These were co-expressed with β2a and $\alpha 2\delta$ subunits in HEK293 cells. In the inside-out mode, α1C and $\alpha 1C\Delta$ showed minimal open-probabilities in a basic internal solution (run-down), whereas $\alpha 1C\Delta$ with CaM and $\alpha 1C\Delta$CaM maintained detectable channel activity, confirming that CaM was necessary, but not sufficient, for channel activity. Previously, we reported that ATP was required to maintain channel activity of α1C. Unlike α1C, the mutant channels did not require ATP for activation in the early phase (3–5 min). However, $\alpha 1C\Delta$ with CaM + ATP and $\alpha 1C\Delta$CaM with ATP maintained activity, even in the late phase (after 7–9 min). These results suggested that CaM and ATP interacted dynamically with the proximal C-terminal tail of the channel and, thereby, produced channel activity. In addition, okadaic acid, a protein phosphatase inhibitor, could substitute for the effects of ATP on α1C but not on the mutant channels. These results supported the hypothesis that CaM and ATP maintain activity of Cav1.2 channels, further indicating that ATP has dual effects. One maintains phosphorylation of the channel and the other becomes apparent when the distal carboxyl-terminal tail is removed.

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Abbreviations AKAP, A-kinase anchoring protein; CaM, calmodulin; DCRD, distal C-terminal regulatory domain; DCT, distal C-terminal region; eDCT, extended DCT; OA, okadaic acid; PCRD, proximal C-terminal regulatory domain; PKA, protein kinase A; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B.

Introduction

L-type Ca^{2+} channels are widely distributed in the plasma membranes of nerve, muscle and secretory cells, and their activation elevates intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), triggering muscle contraction, secretion or gene expression (Bers, 2002; Flavell & Greenberg, 2008; Catterall, 2011; Hofmann *et al*., 2014). The activity of L-type Ca^{2+} channels is regulated by $[Ca^{2+}]_i$ through feedback mechanisms known as Ca^{2+}-dependent facilitation and inactivation (Pate *et al.* 2000; Zuhlke *et al.* 2000; Pitt *et al.* 2001; Erickson *et al.* 2003; Kim *et al.* 2004). These mechanisms require interaction of the Ca^{2+}-binding protein calmodulin (CaM) with the channels (Peterson *et al.* 1999; Qin *et al.* 1999; Zuhlke *et al*., 1999, 2000; Ivanina *et al.* 2000; Erickson *et al*., 2001, 2003; Pitt *et al.* 2001; Tang *et al.* 2003; Kim *et al.* 2004).

CaM has four Ca^{2+}-binding domains (EF-hand motifs). It senses $[Ca^{2+}]_i$, and mediates Ca^{2+}-dependent modification of target proteins, including L-type Ca^{2+} channels. The molecular mechanisms by which CaM regulates L-type Cav1.2 channels have been extensively investigated. Cav1.2 channels have multiple binding sites for CaM, in the N-terminal and I–II linker regions and in pre-IQ and IQ domains of the C-terminal region of the α1C subunit (Kim *et al.* 2004; Zhou *et al.* 2005; Asmara *et al.* 2010; Minobe *et al.* 2011; Ben Johny *et al.* 2013). However, conformations of the channel–CaM complex, in resting, facilitatory and inactivated states, were described differently in various studies and thus remain to be established.

In addition to Ca^{2+}-dependent regulation of channels, CaM plays a crucial role also in maintaining activity of Cav1.2 channels under basal conditions. We previously proposed that basal activity of Cav1.2 channels can be regulated by CaM and ATP, through dynamic interactions. Namely, in the resting state, Cav1.2 channels are not permanently associated with CaM but are, instead, dynamically interacting with free CaM and ATP, with only the CaM- and ATP-bound channels exhibiting channel activity upon depolarization (Xu *et al.* 2004; Han *et al.* 2010; Minobe *et al.* 2011).

In the present study, we used mutant channels, in which the C-terminal tail of the α1C subunit was truncated, with or without a CaM molecule linked to the C-terminal end of the truncated channel (Mori *et al.* 2004). With these mutants, we could further test our hypothesis regarding regulation of the channels by CaM and ATP in the inside-out mode. The results provided new insights into the roles of CaM and ATP in regulating basal activity of Cav1.2 Ca^{2+} channels.

Methods

Plasmid construction and transfection of HEK293 cells

Rabbit α1C subunit (wildtype) and its mutants, and the auxiliary subunits, rabbit β2a and rat $\alpha 2\delta$, were used (Mori *et al.* 2004). In $\alpha 1C\Delta$, the C-terminus was deleted at amino acid (a.a.) position 1671. In $\alpha 1C\Delta$CaM, CaM was fused, through a linker of eight-glycine residues, to the C-terminus of $\alpha 1C\Delta$. HEK293 cells (a generous gift from Dr K. Yamaoka, Hiroshima University) were plated onto glass coverslips in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA) with 10% fetal bovine serum, 100 μg ml^{-1} penicillin and streptomycin (Gibco, Carlsbad, CA, USA). The wildtype and mutant α1C subunits were co-transfected with β2a, $\alpha 2\delta$ and green fluorescent protein in equimolar ratios using Trans-Fast transfection reagent (Promega, Madison, WI, USA), according to the manufacturer's instructions. The cells expressing channels were identified by green fluorescent protein fluorescence through a fluorescence microscope and were used for patch-clamp experiments at 18–48 h after transfection.

Patch-clamp experiments and data analysis

Calcium channel activity was elicited by a depolarizing pulse from a holding potential of −70 mV to 0 mV for 200 ms, at a rate of 0.5 Hz, with a patch clamp amplifier (EPC-7; List, Darmstadt, Germany). The pipette solution contained 50 mM $BaCl_2$, 70 mM tetraethylammonium chloride, 0.5 mM EGTA, 0.003 mM Bay K 8644 (Biomol International LP, Plymouth Meeting, PA, USA) and 10 mM Hepes-CsOH buffer, pH 7.4. The bath solution contained 120 mM potassium aspartate, 30 mM KCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ (free$[Ca^{2+}]$ = 80 nM) and 10 mM Hepes-KOH buffer, pH 7.4. The current signals were filtered at 1–1.5 Hz and fed to a computer at a sampling rate of 3.3 kHz, where the capacity and leakage currents were subtracted digitally. The NPo value was used to represent channel activity, where N is the number of channels in the patch and Po is the time-averaged open state probability of the channels at each depolarizing pulse. NPo was calculated based on the equation $NPo = I/i$, where I is the mean current during the 5–105 ms period after the onset of the test pulses and i is the unitary current amplitude. For comparison, the channel activity recorded in the inside-out mode for 2 min was normalized during recording in the preceding cell-attached mode. Averaged activities, represented by NPo, of wildtype and mutant channels in the cell-attached mode are shown in

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Supporting Information A. Test solutions were applied by moving the patch pipette into a small inlet of the chamber that was connected to the injection system. Based on previous studies (Han *et al.* 2010; Minobe *et al.* 2011), the control activity in the inside-out mode was measured in the presence of 1 μM CaM, 3 mM ATP and 80 nM free Ca^{2+}. The mean *NPo* values were recorded at 3–5 min (early period) and 7–9 min (late period), based on previous observations that the run-down of Cav1.2 channels had two phases, fast and slow, the time constants of which were ∼30 s and ∼3.6 min, respectively (Kameyama *et al.* 1997; Xu *et al.* 2004). In the experiments summarized in Figs 4 and 5, 1 μM okadaic acid (OA; Sigma-Aldrich) was used to inhibit protein phosphatase activity. Data are presented as means ± SEM (number of observations). Student's *t* test, Dunnett's test or ANOVA with *post hoc* Tukey HSD test were used to determine statistically significant differences.

Preparation of calmodulin

Human CaM cDNA (a.a. 3–149) was cloned from HEK293 cells and inserted into a pET-21b vector (Novagen, Madison, WI, USA). The vector was transformed into *Escherichia coli* BL21 (DE3) (Stratagene, La Jolla, CA, USA) and protein expression was induced by 1 mM isopropyl β-thiogalactopyranoside (IPTG). After IPTG induction, the bacterial suspension was treated with 0.1 mg ml^{-1} lysozyme and then sonicated, and the lysate was separated by centrifugation, as previously described (Minobe *et al.* 2011). CaM was purified by hydrophobic interaction chromatography using a Phenyl-Sepharose 6 fast flow column (AKTA prime plus; GE Healthcare, Uppsala, Sweden), according to the manufacturer's instructions. Protein purity was confirmed by electrophoresis on SDS polyacrylamide gels. The concentration was determined by absorbance, using an extinction coefficient at 276 nm (E_{276}) of 0.18 $(mg\ ml^{-1})^{-1}\ cm^{-1}$, measured in a spectrophotometer (NanoDrop2000c; Thermo Fisher, Carlsbad, CA, USA).

Results

CaM interacted dynamically with Cav1.2 channels

Cav1.2 channels were expressed in HEK293 cells with auxiliary β2a and $\alpha 2\delta$ subunits. As shown in Fig. 1*A*, we prepared three types of Cav1.2 channel construct, the wildtype α1C and mutant derivatives, $\alpha 1C\Delta$ and $\alpha 1C\Delta$CaM (Mori *et al.* 2004). The $\alpha 1C\Delta$ was truncated at the extended distal C-terminus (eDCT) region at a.a. 1671. The $\alpha 1C\Delta$CaM had CaM linked to the truncated C-terminus of $\alpha 1C\Delta$ through an eight-glycine linker. The mutants retained important functional sites for channel regulation in the proximal C-terminal tail. These were the Ca^{2+}-binding EF-hand motif (a.a. 1522–1555) (Peterson *et al.* 2000; Brunet *et al.* 2009) and the CaM binding regions, preIQ (a.a. 1599–1639) and IQ regions (a.a. 1648–1668) (Peterson *et al.* 1999; Zuhlke *et al.* 1999; Pitt *et al.* 2001; Dzhura *et al.* 2003; Erickson *et al.* 2003; Tang *et al.* 2003; Kim *et al.* 2004; Brunet *et al.* 2009). However, putative protein kinase A (PKA) phosphorylation sites at the mid-to-distal C-terminal (a.a. 1700 and 1928) (De Jongh *et al.* 1996; Hall *et al.* 2006; Fuller *et al.* 2010) were missing, while one in the proximal C-terminal (a.a. 1575 and 1627) was preserved (Minobe *et al.* 2014). The proximal and distal C-terminal regulatory domains (PCRD, a.a. 1675–1731; and DCRD, a.a. 1822–2171, respectively) (Hulme *et al.* 2006; Fuller *et al.* 2010) were also deleted in the mutant channels.

First, we compared activities of the wildtype and mutant channels in the inside-out mode. After inside-out patch formation, the activity of wildtype α1C was decreased and completely disappeared within a few minutes (Fig. 1*B*); this is known as the run-down of channels. This run-down consisted of fast and slow phases, with time constants of ∼30 s and ∼3.6 min, respectively (Kameyama *et al.* 1997; Xu *et al.* 2004). Therefore, we analysed the open probability of the channels in the early period (3–5 min after the inside-out mode) and late period (7–9 min) and normalized values to those during the cell-attached mode, yielding the relative activity of the channels. The relative activities of α1C were 6.2 ± 2.0 and 1.6 ± 0.8% ($n = 9$) for the early and late periods, respectively. The $\alpha 1C\Delta$ also showed a run-down within a few minutes in the inside-out mode (Fig. 1*C*), with relative activities of 14.1 ± 3.4 and 2.7 ± 0.8% ($n = 11$) in the early and late periods, respectively. Conversely, $\alpha 1C\Delta$CaM maintained its activity in the inside-out mode (Fig. 1*D*), with relative activities of 70 ± 8.1 and 31.2 ± 4% ($n = 13$) in the early and late periods, respectively. Fig. 1*E* summarizes the relative activities of α1C, $\alpha 1C\Delta$ and $\alpha 1C\Delta$CaM in the inside-out mode. $\alpha 1C\Delta$CaM, but not $\alpha 1C\Delta$, showed significantly higher activity than α1C, in both the early and the late periods ($P < 0.001$). These data supported the hypothesis that the run-down phenomenon was caused by dissociation of CaM from the channel. This suggested that CaM was not permanently associated with but, instead, dynamically interacted with the channels. Furthermore, it also suggested that eDCT was unnecessary for the basal activity of the channels.

CaM induced activity of $\alpha 1C\Delta$ in the inside-out mode

The $\alpha 1C\Delta$, similar to wildtype channels, could not maintain channel activity in the inside-out mode with the basic internal solution, as shown in Fig. 1. It was previously shown that externally applied CaM could induce activity in wildtype channels (Xu *et al.* 2004). Therefore, we compared the effects of externally applied

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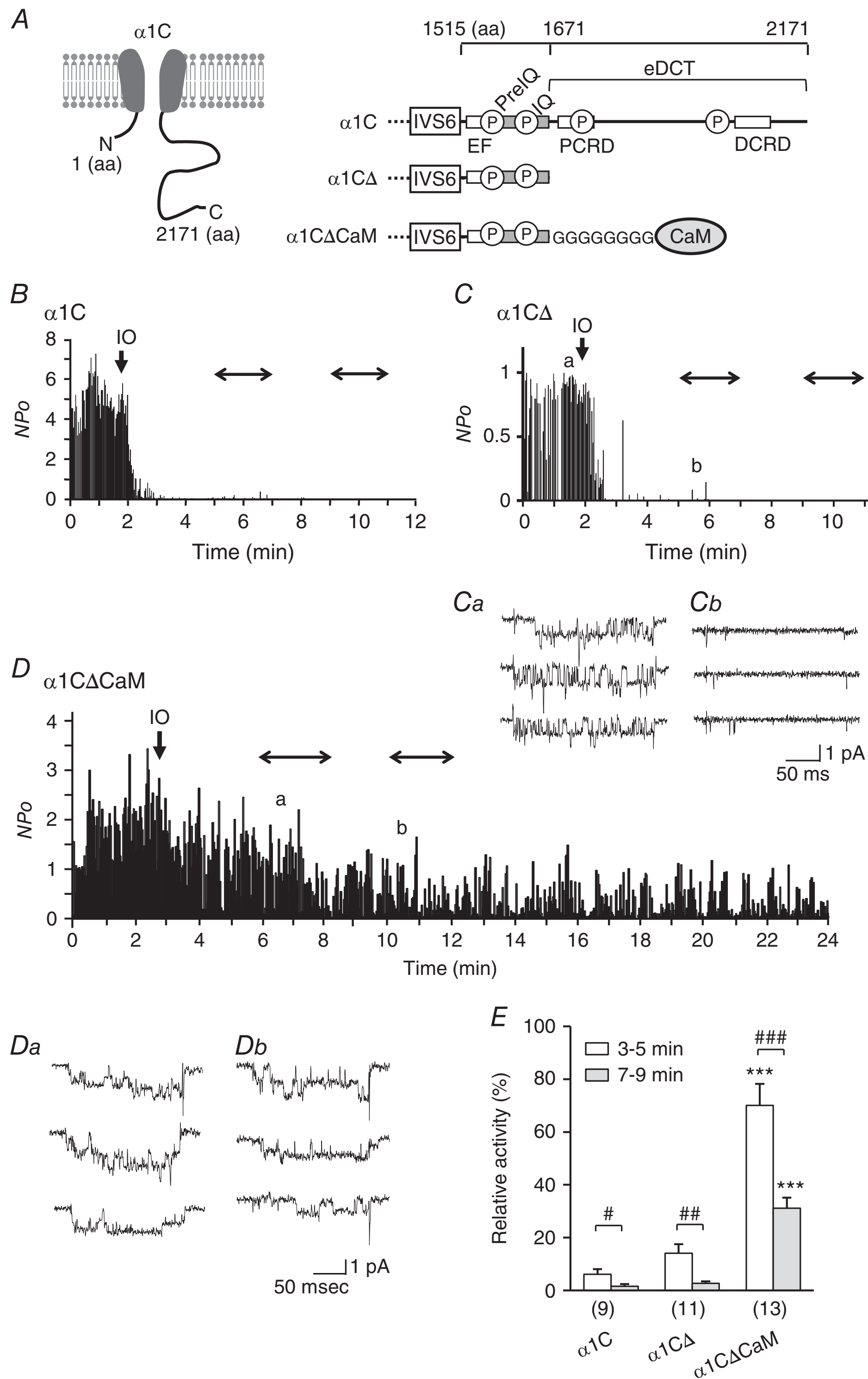

Figure 1. CaM-linked Cav1.2 channels maintained activity in the inside-out mode
A, a schematic illustration of the Cav1.2 channel (*left*) and structures of the mutants, α1CΔ and α1CΔCaM (*right*). Functional sites in the C-terminal tail of α1C (a.a. 1515–2171 in rabbits) are shown: EF-hand motif (EF; a.a. 1522–1555), preIQ (a.a. 1599–1639), IQ (a.a. 1648–1668), proximal and distal C-terminal regulatory domains (PCRD and DCRD; a.a. 1675–1731 and 1822–2171) and putative PKA phosphorylation sites (P; a.a. 1575, 1627, 1700 and 1927). The α1CΔ was truncated at a.a. 1671, which removed PCRD, DCRD and the putative PKA phosphorylation sites. The α1CΔCaM was constructed by connecting CaM to the C-terminus of α1CΔ with an eight-glycine linker. The region including a.a. 1671–2171 is denoted as the extended distal C-terminus (eDCT).

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B–D, the *NPo* value, an indicator of the open-state probability, of each depolarization pulse (0.5 Hz), plotted against time, for α1C (*B*), α1CΔ (*C*) and α1CΔCaM (*D*). After recording in the cell-attached mode, the patch was excised to initiate inside-out mode at the time indicated by an arrow (IO). We measured channel activity in the cell-attached mode for 2 min (control) and then, in the inside-out mode, at 3–5 min (early period) and at 7–9 min (late period), as indicated by the arrows. Representative consequent current traces recorded at the time indicated on the graph are shown in *C* and *D*. *E*, summary of the relative channel activity, normalized by control activity, at 3–5 min (white bar) and 7–9 min (grey bar) after inside-out. Data are means ± SEM. Number of experimental repeats is shown in parentheses. Significant differences are denoted by $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ (by Student's *t* test) or $^{***}P < 0.001$ (*vs* α1C, by Dunnett's test).

CaM on activities of wildtype and α1CΔ channels. When 1 μM CaM was applied to α1C within 1 min after inside-out patch formation, the activity of α1C was decreased (Fig. 2*A*), with relative activities of 6.8 ± 2.0 and 2.5 ± 1.9% ($n = 7$) in the early and late periods, respectively, similar to its activity without CaM (Fig. 2*B*). Interestingly, α1CΔ showed increased activity in the presence of 1 μM CaM (Fig. 2*C*), with relative activities of 60.5 ± 8.9 and 35.4 ± 8.8% ($n = 10$) in the early and late periods, respectively (Fig. 2*D*). These values were significantly higher than those without CaM ($P < 0.001$). Notably, the relative activity of α1CΔ in the presence of CaM was comparable to that of α1CΔCaM (Fig. 1*E*). Furthermore, 1 μM CaM had no additional effect on the activity of α1CΔCaM (data not shown). This suggested that single CaM linked to the channel through the glycine linker was roughly equivalent to 1 μM free CaM, in terms of channel activation. Taken together, α1CΔ had higher channel activity in the presence of CaM than did wildtype channels. This implied that eDCT, which was missing in α1CΔ, might have acted as an antagonist to CaM in promoting channel activity.

ATP increased activity of the Cav1.2 mutants in the inside-out mode

Channel activity was induced in the mutant channels in the inside-out mode when CaM was either covalently

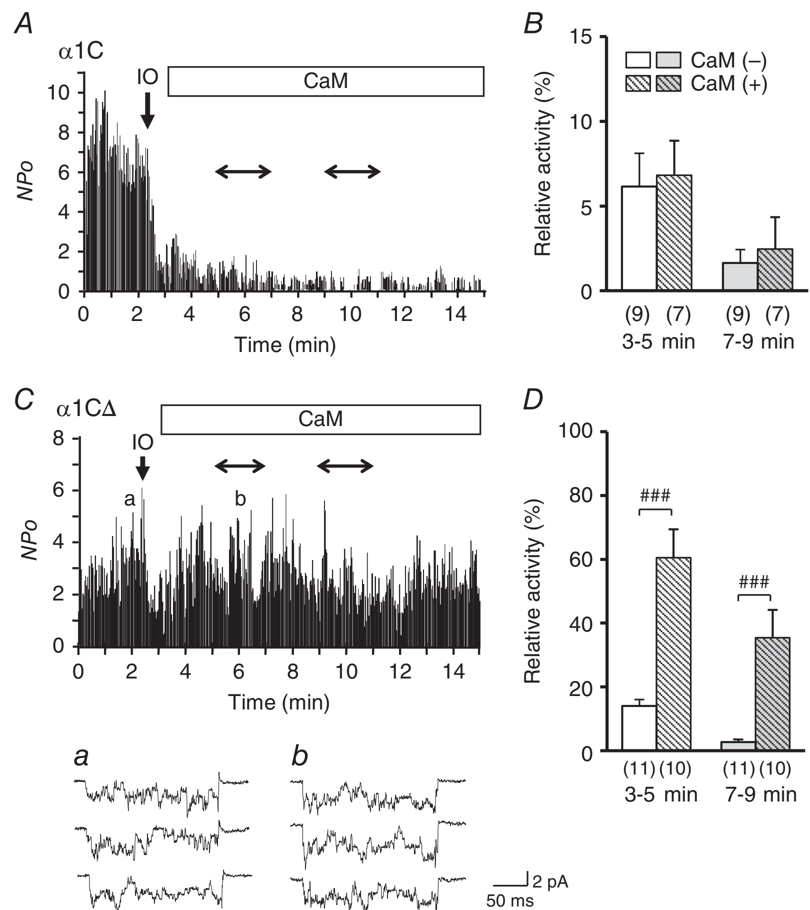

Figure 2. C-terminal truncated but not wildtype α1C maintained activity with CaM alone in the inside-out mode
A and *C*, effects of CaM on α1C (*A*) and α1CΔ (*C*) in the inside-out mode. Test solution containing 1 μM CaM (free [Ca^{2+}] = 80 nM, pH = 7.4) was applied during the time indicated by the rectangle. The *NPo* value was calculated and plotted against time, as for Fig. 1*B–D*. The inside-out mode (IO) was initiated at the time indicated by the arrow. A series of representative current traces recorded at the time indicated on the graph are shown in the lower panel of *C*. *B* and *D*, summary of the relative channel activities in the inside-out mode without CaM (reproduced from Fig. 1*E*) or with CaM (striped bar), measured in the early and late periods (3–5 and 7–9 min, respectively), are shown for wildtype and α1CΔ channels. Data are means ± SEM. Number of experimental repeats is shown in parentheses. Significant differences are denoted by $^{###}P < 0.001$ (by Student's *t* test).

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linked or exogenously applied to the channels. However, channel activity under these conditions was lower than that recorded in the cell-attached mode, especially in the late period of the inside-out mode. In the wildtype channels, ATP is known to enhance channel activity in the presence of CaM (Xu *et al.* 2004). Therefore, we next compared the effects of ATP on wildtype and mutant channels. We first examined the effects of 3 mM ATP without CaM on the activity of α1C and α1CΔ in the inside-out mode. We found that both α1C and α1CΔ had low activity, with relative activities of $5.62 \pm 2.82\%$ ($n = 9$) and $3.37 \pm 2.98\%$ ($n = 3$), respectively, at 3–5 min after the inside-out induction (data not shown). This confirmed that ATP alone did not produce significant channel activity in the inside-out mode (Xu *et al.* 2004). We then tested the effects of CaM and ATP (CaM + ATP) on channel activity. The α1C in the presence of CaM + ATP had relative activities of $38.2 \pm 12.5\%$ ($n = 8$) and $25.6 \pm 5.3\%$ ($n = 10$) in the early and late periods, respectively (Fig. 3*A*). α1CΔ had higher activity in the presence of CaM + ATP than did the wildtype channel, with relative activities of 87.3 ± 10.6 and $66.3 \pm 10.6\%$ ($n = 10$) in the early and late periods, respectively (Fig. 3*B*). The α1CΔCaM with ATP also had high relative activities, at 80.5 ± 6.7 and $73.7 \pm 4.9\%$ ($n = 15$) in the early and late periods, respectively (Fig. 3*C*). As summarized in Fig. 3*D*, activity of α1C was significantly increased in the presence of CaM + ATP, compared with CaM alone ($P < 0.05$). However, it was still significantly lower than those of the mutant channels ($P < 0.01$). Conversely, activity of α1CΔ with CaM + ATP and α1CΔCaM with ATP had similarly high levels of activity (>80%) in the early period. Furthermore, these high levels of activity were maintained in the late period (approximately 70%; $P < 0.05$ *vs* with CaM alone for α1CΔ and $P < 0.001$ *vs* no ATP for α1CΔCaM). Thus, ATP enhanced channel activity in the presence of CaM for all three types of channel. Furthermore, the effects of ATP involved at least two mechanisms, one enhancing CaM-induced activity in the early period and the other inhibiting (or slowing) the run-down in the late period.

OA partly mimicked the action of ATP on Cav1.2 channels

The results described so far showed that ATP enhanced Cav1.2 channel activity in the presence of CaM, in either mutant or wildtype channels. It was reported that ATP affected channel activity in a phosphorylation-independent manner (O'Rourke *et al.* 1992; Yazawa *et al.* 1997) and could bind to the α1C subunit of the channel (Feng *et al.* 2014). Furthermore, a phosphatase inhibitory effect of ATP was reported (Srivastava *et al.* 2002). The eDCT region was shown to anchor protein phosphatases 1 and 2 (PP2A and PP2B, respectively), directly for PP2A (a.a. 1795–1818 and 1965–1971) and indirectly for PP1, via an A-kinase anchoring protein (AKAP) (AKAP-binding domain, a.a. 2057–2115) (Davare *et al.* 2000; Hulme *et al.* 2003; Hall *et al.* 2006; Xu *et al.* 2010; Diviani *et al.* 2011; Le *et al.* 2011).

Thus, we investigated the possibility that ATP might antagonize phosphatase activity that was still attached to the wildtype channels in the inside-out patches. To test this hypothesis, we examined the effects of 1 μM OA (an inhibitor of PP1 and PP2A), instead of ATP, on channel activities in the inside-out mode, with results summarized in Fig. 4. The α1C activity in the presence of CaM + OA was no different from that with CaM + ATP (shown in Fig. 3), the former having relative activities of $43.1 \pm 8.0\%$ ($n = 10$) and $28.0 \pm 7.4\%$ ($n = 8$) in the early and late periods, respectively. There were no additive effects of OA in the presence of CaM and ATP ($43.3 \pm 7.7\%$, $n = 7$, and $29.0 \pm 10.7\%$, $n = 6$, in the early and late periods, respectively), implying that the effects of ATP and OA might, at least in part, share a common mechanism in promoting α1C activity. It should be noted that the mutant derivatives have no phosphatase-anchoring domains. In contrast, OA did not affect α1CΔ or α1CΔCaM activities. The relative activities of α1CΔ in the presence of CaM + OA were 42.0 ± 12.1 and $20.7 \pm 8.0\%$ ($n = 5$) in the early and late periods, respectively, while the corresponding values for α1CΔCaM in the presence of OA were 57.8 ± 8.4 and $27.9 \pm 7.4\%$ ($n = 7$). These values were no different from those with CaM alone (without OA), but were significantly lower than for α1CΔ with CaM + ATP and α1CΔCaM with ATP ($P < 0.05$), particularly in the late period. Furthermore, the relative activities of α1CΔ with CaM + OA + ATP were 77.8 ± 11.0 and $57.0 \pm 11.9\%$ ($n = 5$) and those of α1CΔCaM with OA + ATP were 91.3 ± 11.2 and $87.2 \pm 17.0\%$ ($n = 8$) in the early and late periods, respectively. Again these values were no different from those obtained without OA. These results suggested that the effects of OA were absent or minimal in α1CΔ and α1CΔCaM.

From these data, we hypothesized that OA could substitute for most effects of ATP in wildtype α1C, while it could not do so in the eDCT-deletion mutants. In particular, OA failed to reproduce the activity-enhancing effects of ATP on the mutant channels in the late period of the inside-out mode. The potential involvement of an OA-resistant phosphatase, such as PP2B, was excluded because a PP2B inhibitory mixture (1 μM cyclosporine A + 1 μM cyclosphilin A) could not substitute for ATP (Supporting Information B). This implied that ATP had at least two actions on channel activity. OA could substitute for one of these, and thus it was related to inhibition of phosphatase activity. OA could not substitute for the other, so it has an unknown mechanism.

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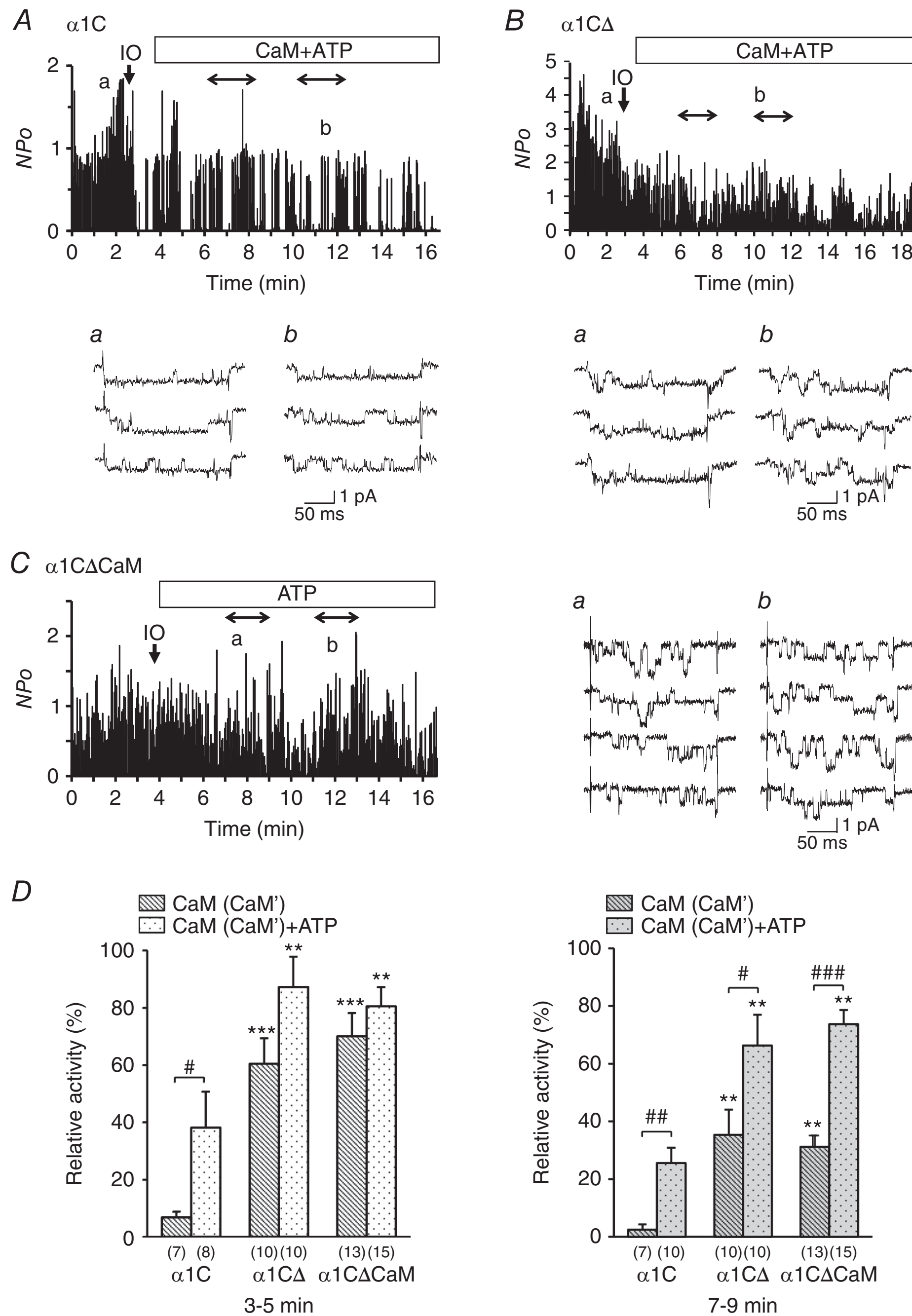

Figure 3. Effects of ATP on channel activity of α1C and its mutants

A–C, effects of CaM and ATP on α1C (*A*) and α1CΔ (*B*), and of ATP on α1CΔCaM (*C*). ATP at 3 mM (free[Ca^{2+}] = 80 nM, pH = 7.4) was applied at the time indicated by the box, along with 1 μM CaM, to α1C (*A*) and α1CΔ (*B*) or, without CaM, to α1CΔCaM (*C*). The *NPo* value was calculated and plotted against time as in Fig. 1*B–D*. Start of the inside-out mode (IO) and the periods to measure channel activity (arrows) are indicated. A series of representative current traces recorded at the time indicated on the graph are shown in the lower panel of *A* and *B*, in the right panel of *C*. *D*, summary of the relative channel activities in the inside-out mode with CaM alone for α1C and α1CΔ; or with no additions for α1CΔCaM (indicated as CaM') (striped bar, reproduced from Fig. 2); and with CaM and ATP for α1C and α1CΔ; or with only ATP for α1CΔCaM (indicated as CaM') (dotted bar). Values are shown for the early (3–5 min, *left graph*) and late (7–9 min, *right graph*) periods. Data are means ± SEM. Number of experimental repeats is shown in parentheses. Significant differences are marked by $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (by Student's *t* test); or $^{**}P < 0.01$, $^{***}P < 0.001$ (*vs* α1C, by Dunnett's test).

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The slow run-down was attenuated in α1CΔ and α1CΔ CaM mutants

The ability of CaM + ATP to recover activity of wildtype Cav1.2 channels slowly declined with time in the inside-out mode when the channels were incubated with a basic internal solution lacking CaM and ATP (slow run-down; Xu *et al.* 2004). To investigate the properties of this slow run-down, the channels were pre-incubated for 5 min with various pre-test solutions followed by 5 min of incubation with test solutions in the inside-out mode. As shown in Fig. 5*A*, a pre-test solution, for example containing ATP (left panel) or basic electrolytes (right panel), was applied after the inside-out formation for 5 min (run-down period), followed by a test solution containing CaM + ATP, and channel activity at 2–4 min during the test incubation was analysed.

As summarized in Fig. 5*Bb*, CaM + ATP failed to re-prime wildtype channels when pre-incubated with the basic internal solution during the run-down period (relative activity of 1.11 ± 0.61%, n = 6). However, when ATP (as shown in Fig. 5*A*, left panel) or CaM was present during the run-down period, wildtype α1C was re-primed by CaM + ATP, with relative activities of 19.1 ± 5.8% (n = 9) (Fig. 5*Bc*) or 17.2 ± 7.7% (n = 7) (Fig. 5*Bd*), respectively. Similarly, OA application during the run-down period and CaM + OA in the test solution (13.7 ± 4.2%, n = 6; Fig. 5*Bf*) was as effective as ATP in re-priming wildtype α1C (Fig. 5*Bc*). These results provided further support that OA could substitute for ATP in preventing the slow run-down of wildtype channels.

Conversely, α1CΔ could be re-primed by CaM + ATP, even when perfused with the basic internal solution during the run-down period (as shown in Fig. 5*A*, right panel). The relative activity was 57.0 ± 11.9% (n = 6) (Fig. 5*Cc*). This value was comparable to that with CaM + ATP during the run-down period (66.32%; Figs 3*D* and 5*Cd*). However, CaM applied alone failed to re-prime α1CΔ channels during the test period (1.77 ± 1.77%, n = 4, Fig. 5*Ca*). The α1CΔCaM channel could maintain a relative activity of 31.2% without ATP (Figs 1*D* and 5*Da*) and of 73.7% with ATP (Figs 3*C* and 5*Dc*). The channel could maintain a relative activity of 67.4 ± 2.5% (n = 5) even when perfused with the basic internal solution during the run-down period (Fig. 5*Db*). This value was comparable to that observed in the presence of ATP during the run-down period (Fig. 5*Dc*).

These results indicated that wildtype α1C channels required the presence of CaM and ATP (or CaM and OA) to be re-primed. Otherwise, an irreversible, or difficult to reverse, slow run-down would proceed. The presence of CaM, ATP and OA during the run-down period prevented the slow run-down. Conversely, α1CΔ and α1CΔCaM channels, which lacked eDCT, did not require ATP or CaM during the run-down period for re-priming.

Discussion

In this study, we confirmed our previous reports that CaM and ATP dynamically interacted with Cav1.2 channels and also supported basal activity of the channels (Yazawa *et al.* 1997; Xu *et al.* 2004; Han *et al.* 2010; Minobe *et al.* 2011; Liu *et al.* 2015). We also added new findings addressing the roles of CaM and ATP in regulating Cav1.2 Ca^{2+} channels, using a C-terminal truncated channel (α1CΔ) and a channel linked with CaM to the truncated C-terminus (α1CΔCaM), in excised patch recording (inside-out mode). Our major findings were: (1) the eDCT (extended distal C-terminal region) was unnecessary for basal activity of Cav1.2 channels; (2) CaM induced channel activity and ATP facilitated the effect of CaM in the mutant channels; (3) the effects of ATP on channel activity involved multiple mechanisms, one related to inhibition

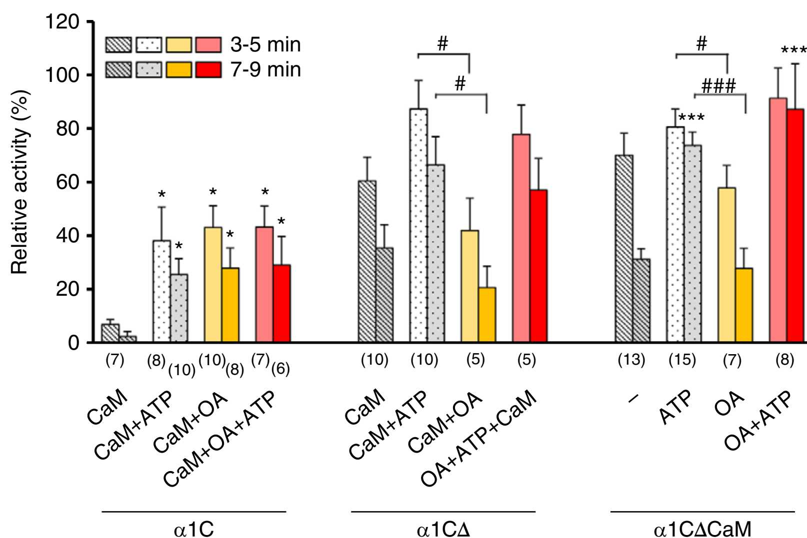

Figure 4. Comparison of effects of ATP and okadaic acid on α1C and its mutants
The relative channel activity in the presence of 1 μM CaM, 3 mM ATP, 1 μM OA and their combinations measured in the early (3–5 min, light bars) and late (7–9 min, dark bars) periods in the inside-out mode. Some data shown were reproduced here from Figs 2*B*, 2*D* and 3*D* for comparison. Data are means ± SEM. The data number is shown in parentheses. Significant differences are marked by #P < 0.05, ###P < 0.001 (by Student's t test) or *P < 0.05, ***P < 0.001 (*vs.* with CaM for α1C and α1CΔ, or *vs* no addition for α1CΔCaM at the corresponding period, by Dunnett's test).

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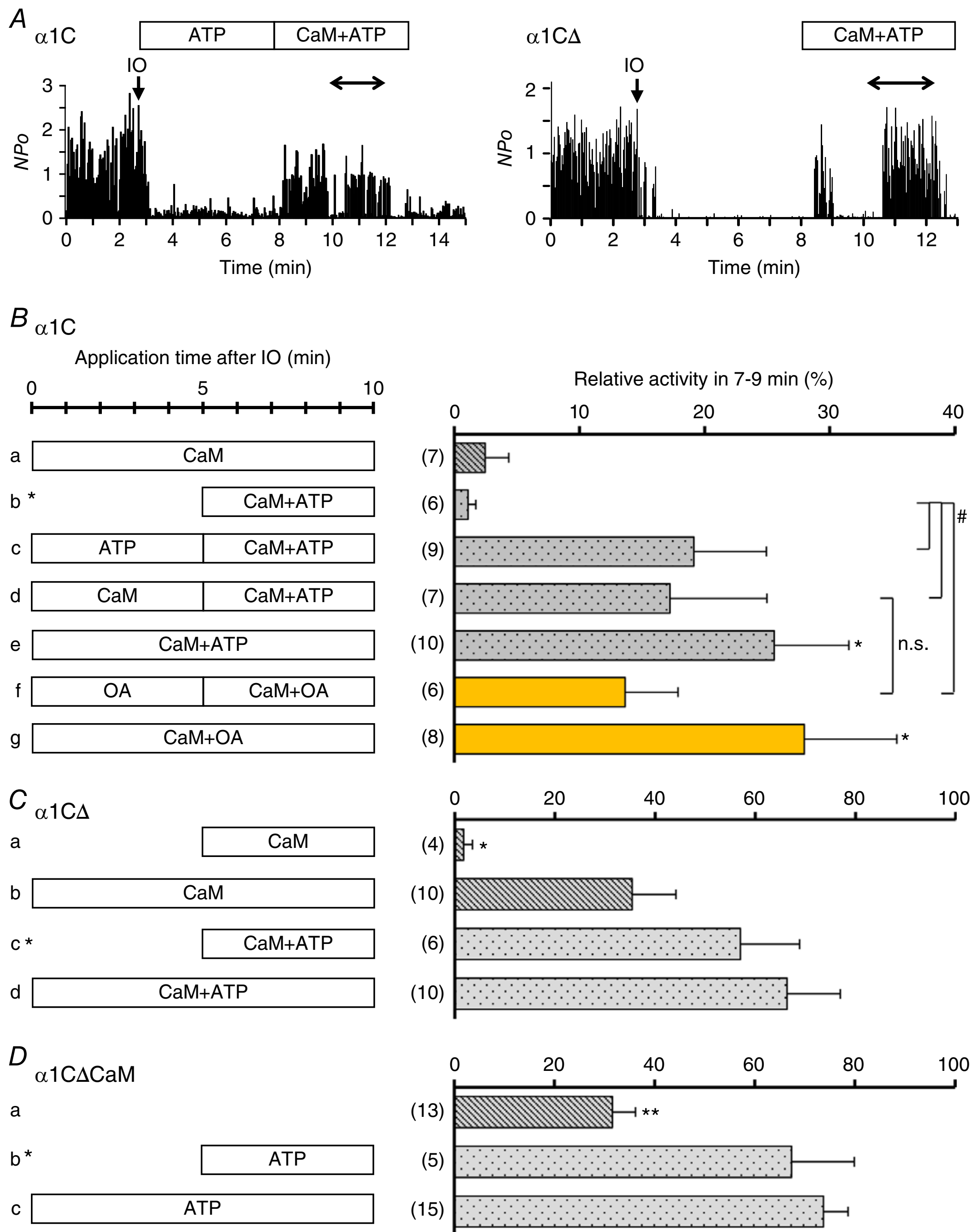

Figure 5. Effects of CaM and ATP on the slow run-down

A, protocol to examine the effects of CaM and ATP on the slow run-down of wildtype (*left*) and α1CΔ (*right*) Cav1.2 channels. After initiating the inside-out mode (IO), the patch was perfused with a conditioning solution (for 5 min) followed by a test solution and the channel activity was measured within 2 min after addition of the test solution (7–9 min after IO). *B–D*, summary of the relative channel activities of α1C (*B*), α1CΔ (*C*) and α1CΔCaM (*D*), with various conditioning and test solutions, as indicated on the left axis. Some data shown were reproduced from Figs 1*E*, 2*B*, 2*D*, 3*D* and 4 for comparison. Data are means ± SEM. The data number is shown in parentheses. Significant differences are denoted by $^{\#}P < 0.05$ and n.s. (not significant) (by Student's *t* test), or $^{*}P < 0.05$, $^{**}P < 0.01$ [*vs* with CaM+ATP for α1C (*Bb**) and α1CΔ (*Cc**), or *vs* with ATP for α1CΔCaM (*Db**), by Dunnett's test].

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of channel dephosphylation and the other not; and (4) the slow run-down of α1CΔCaM was nearly absent in the presence of ATP.

CaM and ATP were required for activity of Cav1.2 channels

In previous studies, we reported that the activity of Cav1.2 channels was abolished in the cell-free mode (run-down), because of loss of CaM or ATP in the cytoplasm. We suggested that CaM and ATP were dynamically interacting with, but not permanently tethered to, the channel (Xu *et al.* 2004; Han *et al.* 2010; Minobe *et al.* 2011). Furthermore, the run-down had two phases, fast and slow (Xu *et al.* 2004). In the present study, we found that both mutants, α1CΔ and α1CΔCaM, exhibited fast and slow run-downs (Figs 1 and 2). In addition, the slow run-down of α1CΔCaM with ATP and α1CΔ with CaM + ATP was decreased compared with that of wildtype α1C in the presence of CaM + ATP (Figs 3 and 4). This provided hints for further investigation of the mechanism of slow run-down in Cav1.2.

The binding site for CaM providing basal activity, or the tethering site, in Cav1.2 channels was proposed to be an IQ region in the proximal C-terminal tail (Pitt *et al.* 2001; Erickson *et al.* 2003; Asmara *et al.* 2010; Liu *et al.* 2010; Minobe *et al.* 2011; Ben-Johny *et al.* 2013). Binding sites for ATP were also localized to the N-terminal and the proximal C-terminal regions (Feng *et al.* 2014). Thus, our findings that CaM and ATP were required even for activity of the eDCT-truncated mutants were consistent with previous studies showing that CaM and ATP acted on a site or sites not located on eDCT.

Effects of ATP on activity of Cav1.2 channels

It was reported that ATP affected channel activity in a phosphorylation-independent manner (O'Rourke *et al.* 1992; Yazawa *et al.* 1997) and that ATP acted as a phosphatase inhibitor (Srivastava *et al.* 2002). The finding that the wildtype α1C had channel activity in the presence of CaM + OA, to a similar extent as that of α1C with CaM + ATP (Fig. 4), suggested that OA, an inhibitor of PP1 and PP2A, can substitute for ATP. This implied that the effects of ATP on channel activity were mediated, at least partially, by inhibition of phosphatase activity. This finding was consistent with an earlier report that OA antagonized the run-down of the channel, in the inside-out mode (Ono & Fozzard, 1992).

Recent reports showed that PP2A bound directly to the distal C-terminal (DCT) region of Cav1.2 channels (Hall *et al.* 2006; Xu *et al.* 2010) and that PP1 was indirectly attached to the DCT through AKAP5 (AKAP79/150; Le *et al.* 2011). These findings suggested that these phosphatases may contribute to regulation of channel activity through dephosphorylation of phosphorylated channels. We recently reported that the slow run-down of wildtype channels was attenuated by inhibition of PP1 or PP2A, but not of PP2B (Xu *et al.* 2016; Yu *et al.* 2016). Reported IC_{50} values of OA for PP1, PP2A and PP2B were 0.27 μM, 2 nM and 3.6 μM, respectively (Herzig & Neumann 2000). Our findings that 1 μM OA affected the activity of wildtype, but not that of α1CΔ or α1CΔCaM channels, both lacking DCT, further supported the idea that PP1 or PP2A attached to the DCT region of α1C can regulate basal activity of Cav1.2 channels (Hall *et al.* 2006; Xu *et al.* 2010; Le *et al.* 2011; Yu *et al.* 2016).

Although phosphorylation of the Cav1.2 channels was required for maintaining basal activity (Herzig & Neumann 2000; duBell & Rogers, 2004), the phosphorylation site responsible for this effect is still unclear. This site is likely to be different from the cAMP–PKA up-modulation site(s). To date, several sites have been suggested as mediating up-modulation of channels by cAMP–PKA signalling, including Ser1928 (rabbit α1C; Gao *et al.* 1997), Ser1700 (Fuller *et al.* 2010; Fu *et al.* 2013; 2014) and Ser1574 (guinea-pig α1C; Minobe *et al.* 2014) of the C-terminal region and certain sites in the β2 subunit (Bünemann *et al.* 1999). However, none of these were established as a requisite site for basal activity of Cav1.2 channels (Weiss *et al.* 2013). The basal activity present in the eDCT-truncated channels suggested that the postulated phosphorylation site may not be located in eDCT, including on Ser1700 or Ser1928. However, the possibility cannot be excluded that the phosphorylation site is located in eDCT, and would act to reverse the inhibitory effect of DCT on channel activity. If true, the eDCT-truncated channel would no longer require this mechanism.

ATP affected channel activity, also in a phosphorylation-independent manner (O'Rourke *et al.* 1992; Yazawa *et al.* 1997), and ATP bound to the α1C subunit of the channel (Feng *et al.* 2014). Our study provided a further clue that ATP can enhance activity of eDCT-deleted channels, and that this effect cannot be mimicked by OA (Fig. 4). Thus, the effects of ATP on channel activity can involve multiple mechanisms, one related to inhibition of dephosphorylation of the channel and the other still unknown. Possible such mechanisms include: (1) ATP bound to the channels maintains them in a conformation that can be re-primed by CaM; and (2) ATP prevents channel degradation, because turnover of L-type Ca^{2+} channels in neurons was reported to be very rapid, with a half-life in hours (Di Biase *et al.* 2011).

Role of the DCT region in channel activity

As we have discussed, the DCT region is involved in regulating channel activity, especially via phosphorylation of the channels. The DCT region contains binding sites for

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AKAP (leucine zipper-like motif, a.a. 2069–2099), which were shown to anchor protein kinases, such as PKA, and phosphatases, such as PP1 (Hulme *et al.* 2003; Diviani *et al.* 2011; Le *et al.* 2011). The DCT region also contains a direct binding site for PP2A (Davare *et al.* 2000; Hall *et al.* 2006; Xu *et al.* 2010). However, activities of the kinases attached to DCT are not absolutely necessary for basal activity of the channel, based on observations that DCT-truncated channels were active (Wei *et al.* 1994; Gerhardstein *et al.* 2000). On the other hand, phosphatases attached to DCT appeared to play a role in the basal activity of the channels. It is possible that the protein kinase required for the basal activity of the channels is not attached to the DCT region. Nevertheless, kinases attached to DCT contributed to regulation of channel activity in response to cellular signals (Catterall, 2011; Hofmann *et al.* 2014).

The DCT region is believed to inhibit channel activity based on findings that DCT-deleted channels showed enhanced currents (Wei *et al.* 1994; Gao *et al.* 2001). We found that the effects of CaM and ATP were enhanced in the α1CΔ, eDCT-deleted mutant (Figs 3 and 4). Thus, it is possible that the inhibitory effect of DCT was mediated by attenuation of the actions of CaM and ATP. It is interesting to note that interaction of the DCRD with PCRD resulted in suppression of Cav1.2 channel activity, and that this interaction was released by phosphorylation of Ser1700 in the PCRD (Hulme *et al.* 2006; Fuller *et al.* 2010). It is speculated that a possible molecular mechanism by which DCRD suppresses channel activity is a change in the interactions of CaM and ATP with the channels. This point should be addressed in future research.

In summary, wildtype and eDCT-truncated mutant channels required CaM and ATP for their activity in the inside-out mode, while the single CaM-linked channel required only ATP. These findings further supported a hypothesis that CaM and ATP are not permanently associated but, instead, dynamically interact with the channels. The effects of ATP appeared to involve two mechanisms, only one related to dephosphorylation. The CaM-linked channel will provide a useful tool for investigating Cav1.2 channels in the inside-out mode, because its fast run-down is prevented by only ATP and its slow run-down is nearly absent.

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Additional information

Competing interests

The authors declare no conflicts of interest.

Author contributions

All experiments were performed in the Department of Physiology at the Kagoshima University. E.M. conceived and designed the project, created figures and wrote the initial manuscript. M.X.M. and M.K. critically reviewed and revised the manuscript and gave final approval of the version to be published.

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Supporting information

The following supporting information is available in the online version of this article.

Comparison of *NPo* values of the expressed channels and effects of PP2A and PP2B inhibitors on the CaM-induced channel activity in inside-out patches.

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